The use of fluorescent indoline dyes for side population analysis.

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Abstract

Dye efflux assay evaluated by flow cytometry is useful for stem cell studies. The side population (SP) cells, characterized by the capacity to efflux Hoechst 33342 dye, have been shown to be enriched for hematopoietic stem cells (HSCs) in bone marrow. In addition, SP cells are isolated from various tissues and cell lines, and are also potential candidates for cancer stem cells. However, ultra violet (UV) light, which is not common for every flow cytometer, is required to excite Hoechst 33342. Here we showed that a fluorescent indoline dye ZMB793 can be excited by 488-nm laser, equipped in almost all the modern flow cytometers, and ZMB793-excluding cells showed SP phenotype. HSCs were exclusively enriched in the ZMB793-excluding cells, while ZMB793 was localized in cytosol of bone marrow lineage cells. The efflux of ZMB793 dye was mediated by ATP binding cassette (ABC) transporter Abcg2. Moreover, staining properties were affected by the side-chain structure of the dyes. These data indicate that the fluorescent dye ZMB793 could be used for the SP cell analysis.

Keywords

flow cytometry, side population, fluorescent dye, hematopoietic stem cells

1. Introduction

The side population (SP) cells, a small population of efficient Hoechst 33342 dye-excluding cells from diverse adult tissues, are highly enriched for stem cell activity [1]. Hoechst 33342 efflux strategies are originally described for the detection of hematopoietic stem cells (HSCs) in murine bone marrow [2, 3]. The bone marrow SP cell population is present in the bone marrow of all species examined [4]. Murine skeletal muscle, brain, testis, mammary gland, lung, heart and embryonic stem (ES) cells also contains SP cells [5-14]. In addition, SP cells were identified in human cancer cells [15-17]. Because of the conserved SP phenotype in a wide range of different types of stem cells, dye efflux assay evaluated by flow cytometry is useful for stem cell studies.

The efflux of Hoechst 33342 was attributed to the member of ATP binding cassette (ABC) transporter of ATP-dependent cell surface proteins. Among various ABC transporter proteins, sub-family G, member 2 (Abcg2; also known as breast cancer resistance protein [Bcrp]) and sub-family B, member 1A/1B (Abcb1a/1b; also known as multidrug resistance [Mdr1a/1b] or P-glycoprotein [P-gp]) are important determinants of the SP phenotype, though the relative contributions of Abcg2 and Abcb1a/1b to the SP phenotype differ in each tissues [18]. In bone marrow, the major determinant of the

SP phenotype is shown to be *Abcg2* through loss- and gain-of-function analyses [12, 19]. *Abcg2* was originally cloned from a breast cancer cell line selected for its unique drug resistance in the presence of a P-gp inhibitor, verapmil [20]. Abcg2 was shown to exclude the chemotherapeutic agent [19], indicating that this transporter may function as a general protectant against endogenous and exogenous substances in stem cells [21].

Ultra violet (UV) light is required for optimal excitation of Hoechst 33342. Recently, another fluorescent Abcg2 substrate DyeCycle Violet (DCV), which can be excited by 405-nm violet light, was reported to identify the almost same population as did Hoechst 33342 [22-24]. However, even though 405-nmdiode laser has become increasingly popular, these UV and violet lights are not as common as 488-nm argon laser in flow cytometer [25]. On the other hand, almost all the modern flow cytometers are equipped with the 488-nm laser, used for excitation of fluorescein isothiocyanate (FITC), green fluorescent protein (GFP), phycoerythrin (PE) and its tandem conjugates, and propidium iodide (PI).

According to the recent report, fluorescent indoline derivative ZMJ018 is recognized as a substrate for the efflux transporters in blood-brain barrier (BBB) and blood-retinal barrier (BRB) and the interaction with efflux transporter may be affected by its substructure [26]. In this study, we identified a fluorescent indoline dye ZMB793,

which is a structurally similar compound of ZMJ018, adequately excited at 488 nm and excluded from murine primitive hematopoietic cells including HSCs. We showed the relationship between SP cells and ZMB793-excluding cells based on double-staining of the cells by ZMB793 and Hoechst 33342. Enrichment of HSCs by ZMB793 efflux assay was evaluated in terms of the surface membrane antigen phenotype and the colony formation activities. We also investigated the intracellular localization of ZMB793 by confocal microscopy. The active efflux of ZMB793 from bone marrow cells was investigated by examining the effect of ABC transporter inhibitors. Furthermore, the change in the staining property dependent on the side-chain structure of indoline dyes was tested by using several analogues of ZMB793.

2. Materials and Methods

2.1. Materials

Fetal bovine serums (FBS) were purchased from Hyclone (Logan, UT, USA) and Stem cell technologies (Vancouver, BC, Canada). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from nacalaitesque (Kyoto, Japan). The monoclonal antibodies 2B8 (anti-c-Kit), D7

(anti-Sca-1), RAM34 (anti-CD34) were purchased from BD Bioscience (Rockville, MD, USA), HM48-1 (anti-CD48), TC15-12F12.2 (anti-CD150), 145-2C11 (anti-CD3e), 53-6.7 (CD8a), RA3-6B2 (anti-B220), 1A8 (anti-Ly6G), M1/70 (anti CD11b) were from BioLegend (San Diego, CA, USA). TER119 (anti-erythrocyte-specific antigen) and human erythropoietin were purchased from eBioscience (San Diego, CA, USA). TO-PRO 3 was purchased from Invitrogen (Carlsbad, CA, USA). 2.4G2 hybridoma was purchased from American Type Culture Collection (Rockville, MD, USA). Fumitremorgan C (FTC), digoxin, and probenecid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). S-clone SF-O3 medium was purchased from Sanko Junyaku Co. (Tokyo, Japan). Bovine serum albumin and β-mercaptoethanol were from Sigma-Aldrich Co. (St. Louis, MO, USA). Mouse stem cell factor (SCF), Human thrombopoietin, mouse interleukin (IL)-3, and human erythropoietin were from R&D systems Inc. (Minneapolis, MN, USA).

2.2. Mice

C57BL/6NCrSlc female mice were purchased at 6-8 weeks old from Shimizu Laboratory Supplies Co. (Kyoto, Japan) and were used between 8 and 12 weeks old. All animal experimentation was conducted in accordance with the guidance of the Institute

for Frontier Medical Sciences, Kyoto University.

2.3. Fluorescent indoline derivatives

All the fluorescent indoline dyes examined were obtained from Canon Inc. (Tokyo, Japan). Stock solutions of the fluorescent indoline dyes were prepared by dissolution in dimethyl sulfoxide (DMSO) at 1mM. The excitation wavelength and the fluorescence wavelength of the fluorescent dyes were obtained by measuring DMSO solution containing 5μM fluorescent dyes by FL4500 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan).

2.4. Preparation of bone marrow cells

Fresh bone marrow cells were harvested from femurs and tibias, and were suspended in Hanks-balanced salt solution (HBSS) supplemented with 2% FBS (Hyclone), 10 mM HEPES, and penicillin/streptomycin (HBSS+). The cell suspensions were filtered through a cell strainer to remove debris. The filtrate was suspended in ice-cold HBSS+ and then pelleted by centrifugation for 5 min at 4 °C. The bone marrow cells were resuspended at 1 x 10^6 cells/ml in HBSS+.

2.5. Dye labeling

Mouse bone marrow cells were resuspended at 10^6 cells/ml in HBSS+ and labeled with 0.01 - 1 μM ZMB793 or its analogues in HBSS+ at 37 °C for 5 - 60 min. For the inhibitor experiments, FTC, digoxin, or probenecid was added to cells at indicated concentration. 1 μM ZMB793 was then added and incubated at 37 °C for 30 min. In other experiments, cells were incubated in the presence of Hoechst 33342 at 37 °C for 30 min. Then ZMB793 were added to give final concentration of 1 μM and incubated at 37 °C for additional 30 min. After staining, the cells were washed and resuspended in ice-cold HBSS+.

2.6. Flow cytometry

For immunostaining, the single-cell suspensions were blocked by the culture supernatant of 2.4G2 (anti-CD16/CD32) hybridoma, and stained with monoclonal antibodies in PBS containing 2% FBS (Hyclone) and 0.1% sodium aside. TO-PRO 3 was used to distinguish dead cells from viable cells. The immuno-stained cells were analyzed on a FACSCanto II flow cytometer equipped with 488-nm, 633-nm, and 405-nm laser or sorted on a FACSAria II cell sorter equipped with 488-nm, 532-nm, 640-nm, 405-nm, and 355-nm lasers (BD Biosciences). Analysis was performed by BD

FACSDiva software (BD Bioscience) and FLOWJO software (Tree Star, San Carlos, CA).

2.7. Confocal microscopy

The bone marrow cells labeled with ZMB793 and TO-PRO 3 were sorted directly onto four-well chambered cover glasses (Lab-Tek™ Chambered Cover glass, NalgeNunc International, Rochester, NY, USA). Confocal microscopy was performed on an LSM 510 META using a 10x/0.3 NA or 63x/1.4 NA oil immersion objective lens (Carl Zeiss, Oberkochen, Germany). The cells were excited by 488-nm laser, and LP650 filter was used to detect fluorescence emission of ZMB793. All acquired images were processed with the LSM Image Browser (Carl Zeiss).

2.8. Single-cell colony assay

The single-cell colony assay was performed as described previously [27]. Briefly, the cells were sorted clonally into 96-well plates containing 100 μl of S-clone SF-O3 medium supplemented with 10% FBS (Stem cell technologies), 1% bovine serum albumin, 10 ng/ml mouse SCF, 10 ng/ml human thrombopoietin, 10 ng/ml mouse IL-3, 1 U/ml human erythropoietin, and 5 x 10^{-5} M β-mercaptoethanol. After 14 days,

colonies containing more than 1000 cells were identified microscopically (n = 3 for each cell type).

2.9. Statistic analysis

All the results were expressed as the mean ± standard deviation (SD). Significant analysis between the experiment groups was done based on the one-way ANOVA, and the difference was considered to be significant at $p < 0.05$.

3. Results

3.1. Characteristics of fluorescent indoline dyes

Figure 1A shows the chemical structures of ZMB793 (molecular weight, 621.79). Figure 1B shows the excitation and emission spectra of ZMB793 in DMSO. ZMB793 was strongly excited at the wavelength of 480-580 nm, and its emission was efficiently detectable through filters passing wavelengths longer than 600 nm.

3.2. Exclusion of fluorescent indoline dye by bone marrow primitive hematopoietic cells

The uptake of fluorescent indoline dye ZMB793 into viable cells was evaluated by flow cytometric analysis. Figure 2A and 2B show the ZMB793 fluorescence in whole mouse bone marrow cells consisting of erythroid, myeloid, lymphoid and primitive hematopoietic cells. A complex fluorescence signal was observed when its fluorescence was detected at two different emission wavelengths, namely yellow (585/42BP) and red (670LP) (Figure 2A). Dose-dependent change in the fluorescence intensity was shown in Figure 2B. At the optimized dye concentration of 1 μM, the population of cells with low fluorescence intensity was characterized as c-Kit^{+}, indicating that these populations were primitive hematopoietic cells including HSCs (Figure 2B). Figure 2C shows the

time-course of the changes of fluorescence intensity of both c-Kit^{+} and c-Kit^{-} cells in bone marrow. The signal intensity profile in c-Kit^{+} cells were nearly flat over a long period of time, whereas the intensity in c-Kit^{-} cells increased gradually and reached to plateau within 30 min.

3.3. ZMB793-excluding cells exhibit SP phenotype

To investigate the relationship between ZMB793-excluding cells and SP cells, double-staining flow cytometric analysis was performed on bone marrow cells (Figure 3). Double-staining analysis of bone marrow cells with ZMB793 and Hoechst 33342 revealed that ZMB793-excluding cells showed an SP phenotype (Figure 3A and 3B), and ZMB793-excluding cells were correlated with Hoechst$^{low/-}$ SP cells (Figure 3C). It was confirmed that both Hoechst 33342 and ZMB793 did not affect the staining of each other (Figure 3D).

3.4. Enrichment of HSCs by ZMB793

Figure 4 shows representative flow cytometric profiles of bone marrow HSCs stained with ZMB793. More than 94% of CD34$^{low/-}$c-Kit^{+}Sca-1^{+}lineage (Lin)$^{-}$ (CD34^{-}KSL) cells, which are highly enriched in HSCs [28], were enriched in the ZMB793-excluding

cells (Figure 4A), though whole Lin^- cells in bone marrow contain only 9% of the dye-excluding cells (Figure 4C). In addition, approximately 90% of $CD48^-CD150^+$ cells, which are also significantly enriched in HSCs [29], were concentrated in the same fraction (Figure 4B).

Figure 5A shows the flow cytometric profile and sort gate definitions for *in vitro* single cell-based colony assay. Figure 5B shows frequencies of colony formation after 14 days of culture. The cells with myeloid and erythroid colony formation ability were detected in ZMB793-excluding cells, whereas no colony was detected in ZMB793 positive population.

3.5. Intracellular localization of fluorescent indoline dye in whole mouse bone marrow cells

Whole mouse bone marrow cells stained with ZMB793 were separated into two populations, ZMB793-positive and -negative fraction, by flow cytometry. Figure 6A and 6B shows the fluorescent images of the sorted bone marrow cells. ZMB793 fluorescent signals were localized in the cytoplasm of the ZMB793-positive cells. (Figure 6A). ZMB793 appeared to be excluded from the kidney bean-shaped nuclear structure typical of monocytes, segmented nuclear structure typical of neutrophils, and large spherical

nuclear structure typical of lymphoid cells (Figure 6A). On the other hand, very little fluorescent signal was detected in the ZMB793-negative fraction, as expected (Figure 6B).

3.6. Elimination of the population of cells with low fluorescence intensity by ABC transporter inhibitors

Figure 7 shows the ZMB793 fluorescence in whole bone marrow cells in the presence or absence of the ABC transporter inhibitors at the concentrations indicated. The cytotoxicity of these inhibitors was evaluated by flow cytometric analysis after TO-PRO 3 labeling, and the maximum concentration set without cytotoxicity were used for the assay (Data not shown). The decrease in the ZMB793 intensity in c-Kit^{+} primitive cells was inhibited by FTC, a selective Abcg2 inhibitor, in dose-dependent manner (Figure 7). Furthermore, the result was supported by the same effect of digoxin, which inhibit Abcg2-mediated efflux, though the specificities are not particularly high (Figure 7). By contrast, probenecid, a nonspecific inhibitor of several ABC-transporters of the Abcc/Mrp subfamily [30], had relatively little effect on the signal intensity of ZMB793 (data not shown).

3.7. Structure dependency of indoline dyes on the uptake and efflux by mouse bone marrow cells

Figure 8 shows the change in the staining property of bone marrow cells dependent on the side-chain structure of indoline dyes. We firstly confirmed that all the ZMB793 analogues used in this experiment had the same excitation and emission spectra patterns as original ZMB793 (Data not shown). Fluorescent intensity changed dependent on the length of the alkyl group attached to one of the side-chain (R_1), and the efflux of indoline dyes from c-Kit$^+$ primitive hematopoietic cells was diminished at the maximum length of alkyl group examined (C5). Staining properties were also affected by the structure of another side-chain (R_2). The efflux of the ZMB793 analogue bearing 6-Methoxy-2-naphtyl (6-MeO-2-Np) group from c-Kit$^+$ primitive hematopoietic cells was diminished. The ZMB793 analogue bearing ethyl group stained bone marrow cells with a broad range. On the other hand, the bone marrow cells stained with ZMB793 analogue bearing 4-(2,2-diphenylethenyl)phenyl group showed minimal staining intensity.

4. Discussion

In the current study, we found that a fluorescent indoline dye ZMB793, excited at 488 nm, was localized in cytosol of bone marrow lineage cells, and excluded from mouse primitive hematopoietic cells including HSCs. $CD34^{-}KSL$ and $CD48^{-}CD150^{+}$ cells in bone marrow were exclusively enriched in the ZMB793-excluding cells. Moreover, the cells with colony formation ability were also exclusively enriched in the ZMB793-excluding cell fraction. The active efflux of ZMB793 dye was inhibited by the specific Abcg2 inhibitor and some non-specific ABC transporter inhibitors.

In this trial, we applied the supravital dye of ZMB793 to the functional assay of efflux in living mammalian cells. We attempted to identify fluorescent dye with its ability to be effluxed from $c\text{-}Kit^{+}$ primitive hematopoietic cells in mouse bone marrow. Several studies have shown that c-Kit is highly expressed on primitive mouse hematopoietic cells including hematopoietic stem cells, early lymphoid progenitors, common myeloid progenitors, granulocyte/monocyte progenitor, and megakaryocyte/erythroid progenitor, but not on most of lineage cells in bone marrow [31-34].

The major advantage of this fluorescent indoline dye is that the dye can be excited at 488 nm. Although the SP cells identified by Hoechst 33342 are useful to

detect primitive stem-like cells in various organs and tissues, UV source is exclusively used to excite Hoechst 33342 [1, 3]. 405-nm excitation source is required for DCV, alternative fluorescent dye to Hoechst 33342. However, UV and 405 nm laser illumination are not as common as 488-, and 633-nm laser in flow cytometer. Thus we screened various fluorescent dyes with respect to their ability to be excited by 488-nm laser, which is the most common laser equipped in flow cytometer. The described method here allows for the dye efflux assay by every known flow cytometers. The excitation spectrum of the indoline dye showed that the indoline dye can be excited at 488 nm strongly enough to be detected by flow cytometer, though this dye is excited most effectively at 544 nm, and that this dye can be combined with immunofluorescent staining of cell surface antigens for flow cytometric analysis. The emission spectrum of this dye showed that fluorescent signal of the dye can be efficiently detected using the long-path filter for Peridinin-chlorophyll-protein complex (PerCP; 650LP), or band-path filters for phycoerythrin cyanine 5 (PE-Cy5; 660/20nm), PerCP or its tandem fluorochrome PerCP-cyanine 5.5 (PerCP-Cy5.5; 710/50 nm).

For SP cell analysis, dual-wavelength flow cytometric analysis is performed. Hoechst 33342-labeled cells are usually analyzed simultaneously through blue and red emission filtersin a linear mode [3, 4]. In this study, ZMB793 fluorescence of whole

bone marrow cells was detected at two different emission wavelengths, red and yellow. This characteristic fluorescence pattern may be caused by the change of emission spectrum of ZMB793 upon binding to some components in bone marrow cells, as previously reported that the fluorescence characteristics of Hoechst 33258 change on binding with DNA [35, 36]. Confocal microscopy revealed that ZMB793 was localized in cytosol of bone marrow cells stained. It is unclear whether this dye bounds with subcellular components or freely diffusing. Further studies are needed to define the molecular state of ZMB793 in cytosol.

Use of a combination of some cell surface antigens has been shown to be useful to isolate HSCs from other cells in murine bone marrow. $CD34^-$ cells, $CD150^+CD48^-$ cells, and $Thy\text{-}1^{low}Flt\text{-}3^-$ cells in KSL cells are enriched in HSCs [28, 29, 37]. In this study, immunofluorescent staining of CD34, c-Kit, Sca-1, CD48, CD150 and Lin such as granulocyte, monocyte, erythrocyte, B cell and T cell markers were combined with ZMB793 staining, and analyzed by flow cytometer. Both $CD34^-$KSL and $CD150^+CD48^-$ cells were enriched in ZMB793-excluding cells. As seen from the above, the combination of indoline dye and immunofluorescent staining is particularly desirable for studies of the cells for which some surface marker antibodies are available.

Single cell-based *in vitro* colony assay was performed for clonal analysis of

HSC colony formation potentials. This approach is the combination of conventional *in vitro* colony assay and fluorescence-activated clone-sorting technology [38, 39]. It is reported that HSCs can give rise to colonies in the presence of appropriate cytokines [38]. We evaluated the number of colonies after 14 days in culture, because most mouse HSCs take approximately 14 days to form fully differentiated colonies, but progenitor cells take 5-10 days to form colonies [39]. We found that colony formation was observed only in ZMB793-excluding cell population. Combined with the results of immunofluorescent analysis, it is likely that HSCs were enriched in ZMB793-excluding cells, though it is unclear whether all the colonies observed were derived from HSCs or some were from progenitor cells. In addition, it was shown that ZMB793-excluding cells and SP cells overlapped one another. It is now clear that dye efflux assay using ZMB793 is useful to evaluate primitive hematopoietic cells including HSCs.

In this study, ZMB793 efflux was inhibited by some ABC transporter inhibitors. FTC completely inhibited the efflux of ZMB793 from c-Kit$^+$ primitive hematopoietic cells in mouse bone marrow. A mycotoxin FTC was identified to reverse drug resistance in a mitoxantrone-selected human colon carcinoma cell line (S1-M1-3.2) that does not overexpress ABC transporters of Abcc (Mrp) subfamily nor Abcba, also known as P-gp/Mdr1 [40]. It almost completely reverses resistance mediated by Abcg2 *in vitro*

[41]. Digoxin also inhibited the efflux of ZMB793 in the same manner as FTC. Digoxin is a cardiac glycoside with a steroid core structure [42]. Although it is known to inhibit a variety of transporters including Organic anion transporting polypeptide (Oatp)2 [43], it also inhibited Abcg2-mediated transport of mitoxantrone [42]. It is well known that Abcg2 is highly expressed on primitive hematopoietic cells including HSCs in murine bone marrow [12, 19]. These suggest that Abcg2 is the major transporter to mediate ZMB793 efflux from c-Kit$^+$ primitive hematopoietic cells.

We have shown that the staining property of indoline dye was changed dependent on the side-chain structure. In our preliminary study, we have examined wider variety of R_1 side-chain structure including single rhodanine ring on R_1 such as ZMJ018. However, only those indoline dyes having dual rhodanine ring showed significant effect to be excluded from HSCs. The efflux of the ZMB793 analogue from c-Kit$^+$ cells was diminished by the elongation of the alkyl group attached to rodanine ring (R_1). On the other hand, ZMB793 analogue with 4-(2,2-diphenylethenyl)phenyl group lost its staining ability. Since 4-(2,2-diphenylethenyl)phenyl group is hydrophobic, the quantity of soluble dye in the staining solution might be low. Other than ZMB793 analogue containing 4-(2,2-diphenylethenyl)phenyl group, the staining heterogeneity of c-Kit$^-$ and c-Kit$^+$ cells was significantly affected by the R_2 side-chain. This result

suggests that the efflux transporter interacting with ZMB793 analogues is same between c-Kit$^+$ cells and c-Kit$^-$ cells. If this was the case, the expression level of the efflux transporter in c-Kit$^+$ cells may be higher. These data indicate that cellular uptake and efflux can be modified by chemically changing side-chain structure.

5. Conclusion

The present study demonstrated that dye efflux assay using ZMB793 is useful to enrich primitive hematopoietic cells including HSCs in mouse bone marrow. 488-nm laser, the most common laser equipped on flow cytometers, is suboptimal for the excitation of ZMB793 fluorescent dye. It was indicated that ZMB793 is effective in animal species where cell surface antibodies to recognize HSCs are not available and in various tissues/organs where antibodies to permit enrichment of stem-like cells.

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Figure 1. Structure and excitation/emission spectra of ZMB793 dye. (A) Chemical structure of ZMB793. (B) Excitation (dashed line) and emission (solid line) spectra of ZMB793 measured at 5 μM in DMSO.

Figure 2. ZMB793 fluorescence in whole mouse bone marrow cells. (A) Representative flow cytometric profile of ZMB793 fluorescence in bone marrow cells. ZMB793 fluorescence was detected at two different filters select the emission wavelengths, namely yellow and red. (B) Flow cytometric profiles of ZMB793 fluorescence in bone marrow cells at indicated concentrations. Data are representatives of three independent experiments. (C) Time-dependent changes in ZMB793 red fluorescence in c-Kit$^-$ bone marrow cells (○) and bone marrow c-Kit$^+$ bone marrow cells (△). Error bars represent SD of the mean. $^*p$ <0.05, significant against the group of c-Kit$^-$ cell fraction.

Figure 3. SP phenotype of ZMB793-excluding cells in mouse bone marrow. Bone marrow cells were stained with ZMB793 and Hoechst 33342, and analyzed by flow cytometer. (A) Flow cytometric profiles of bone marrow cells stained with ZMB793 and Hoechst 33342. ZMB793-excluding cells were gated and plotted for SP cell analysis. (B) Flow cytometric profile of bone marrow cells stained with Hoechst 33342, and plotted for SP cell analysis. (C) Flow cytometric profiles of bone marrow cells stained with ZMB793 and Hoechst 33342 plotted for the parameters of Hoechst-Blue and ZMB793-Red. (D) Flow cytometric profiles of bone marrow cells stained with Hoechst 33342 and plotted for ZMB793 analysis (left) and flow cytometric

profiles of bone marrow cells stained with ZMB793 and plotted for SP cell analysis (right). Data are representatives of three independent experiments.

Figure 4. Enrichment of mouse hematopoietic stem cells in ZMB793-excluding cells. Bone marrow cells were stained with ZMB793 and then immunostained with fluorophore conjugated antibodies. (A) Flow cytometric profiles of bone marrow cells stained with ZMB793 and monoclonal antibodies to isolate $CD34^{-}KSL$. (B) Flow cytometric profiles of bone marrow cells stained with ZMB793 and monoclonal antibodies to isolate $CD48^{-}CD150^{+}$ cells. (C) Flow cytometric profile of bone marrow cells gated as lin^{-} negative fraction. Data are representatives of three independent experiments.

Figure 5. Enrichment of the cells with colony forming ability in ZMB793-excluding cells. Bone marrow cells were stained with ZMB793 and sorted for single cell-based colony assay. (A) Sorting gate of ZMB793-positive cells and -negative bone marrow cells. (B) Number of colonies with >1,000 cells observed after 14 days of culture in ZMB793-positive and -negative bone marrow fractions. No colony was detected in ZMB793-positive fractions. Error bars represent SD of the mean.

Figure 6. Localization of ZMB793 in bone marrow cells. Bone marrow cells were stained with ZMB793 and sorted as ZMB793-positive or -negative fractions. The sorted cells were analyzed with confocal microscopy

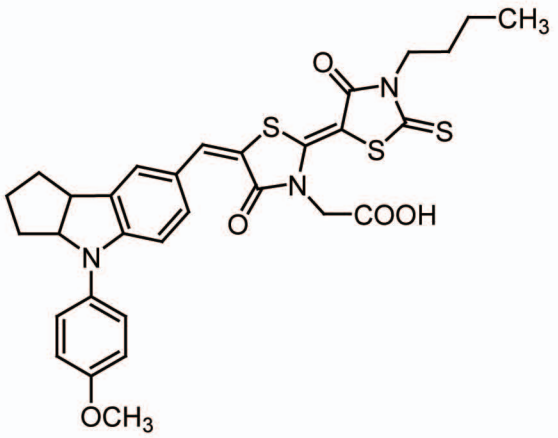
with excitation at 488 nm and emission collected through long-path filter 650LP. (A) Representative confocal and corresponding differential interference contrast (DIC) images of bone marrow cells stained with ZMB793 and sorted as ZMB793-positive fraction. Scale bar = 2 μm. (B) Representative confocal and corresponding differential interference contrast (DIC) images of bone marrow stained with ZMB793 and sorted as ZMB793-negative fraction. Scale bar = 2 μm.

Figure 7. Effect of ABC transporter inhibitors on the efflux of ZMB793 from primitive hematopoietic cells. (A) Representative flow cytometric profile of bone marrow cells stained with ZMB793 and anti-c-Kit without any inhibitors. (B) Flow cytometric profiles of bone marrow cells stained with ZMB793 in combination with the Fumitremorgan C (FTC) or digoxin at indicated concentrations. The bone marrow cells were stained with ZMB793 and then immunostained with anti-c-Kit. Data are representatives of three independent experiments.

Figure 8. Effect of side chains structures on the efflux of ZMB793 from mouse primitive hematopoietic cells. Flow cytometric profiles of bone marrow cells stained with various ZMB793 analogues are shown. Data are representatives of three different experiments.

Fig. 1

A



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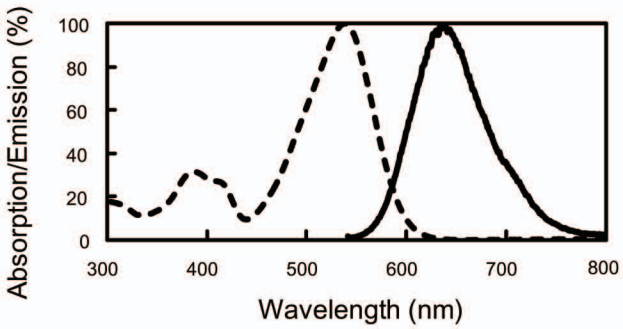

Fig. 2

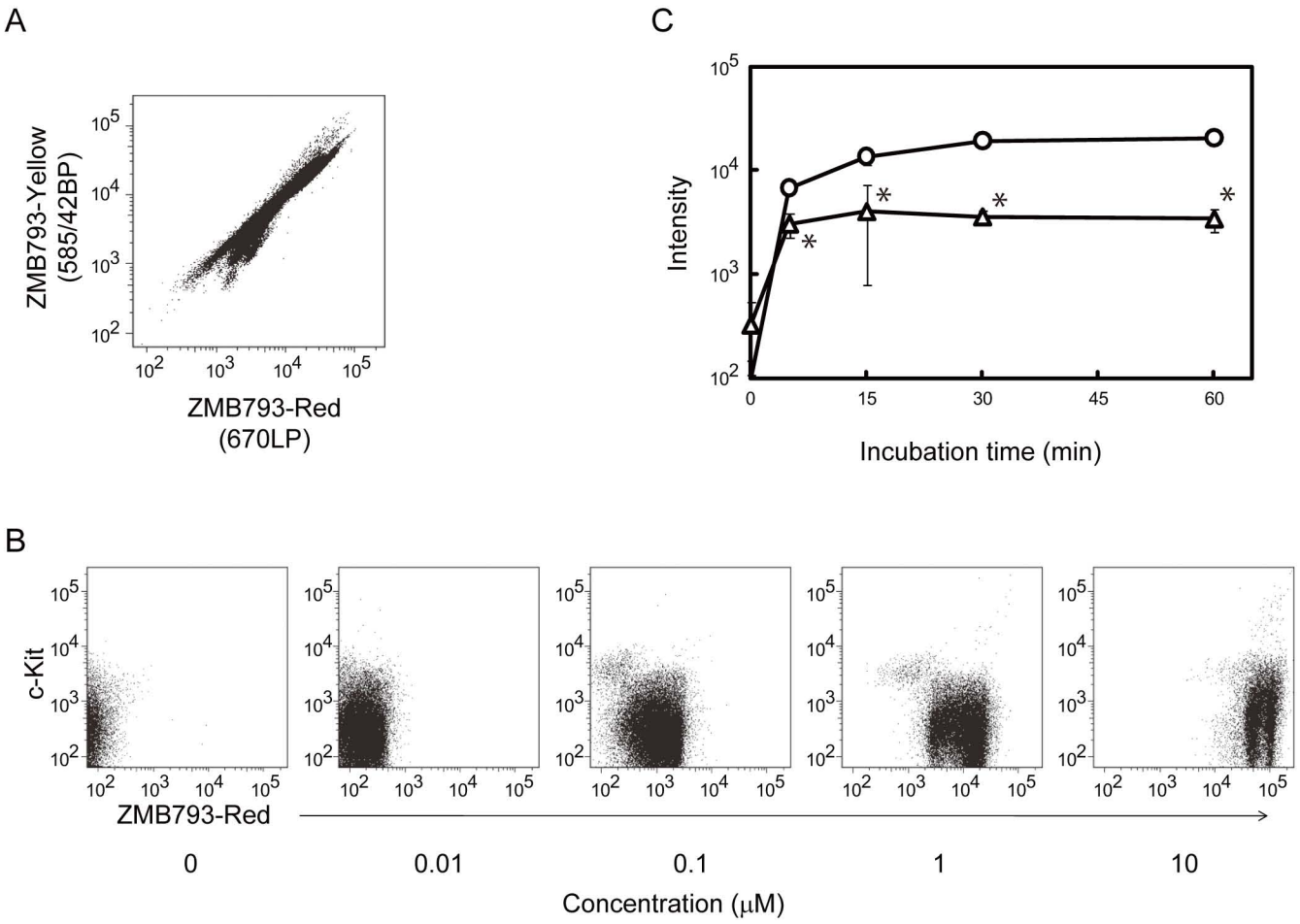

Fig. 3

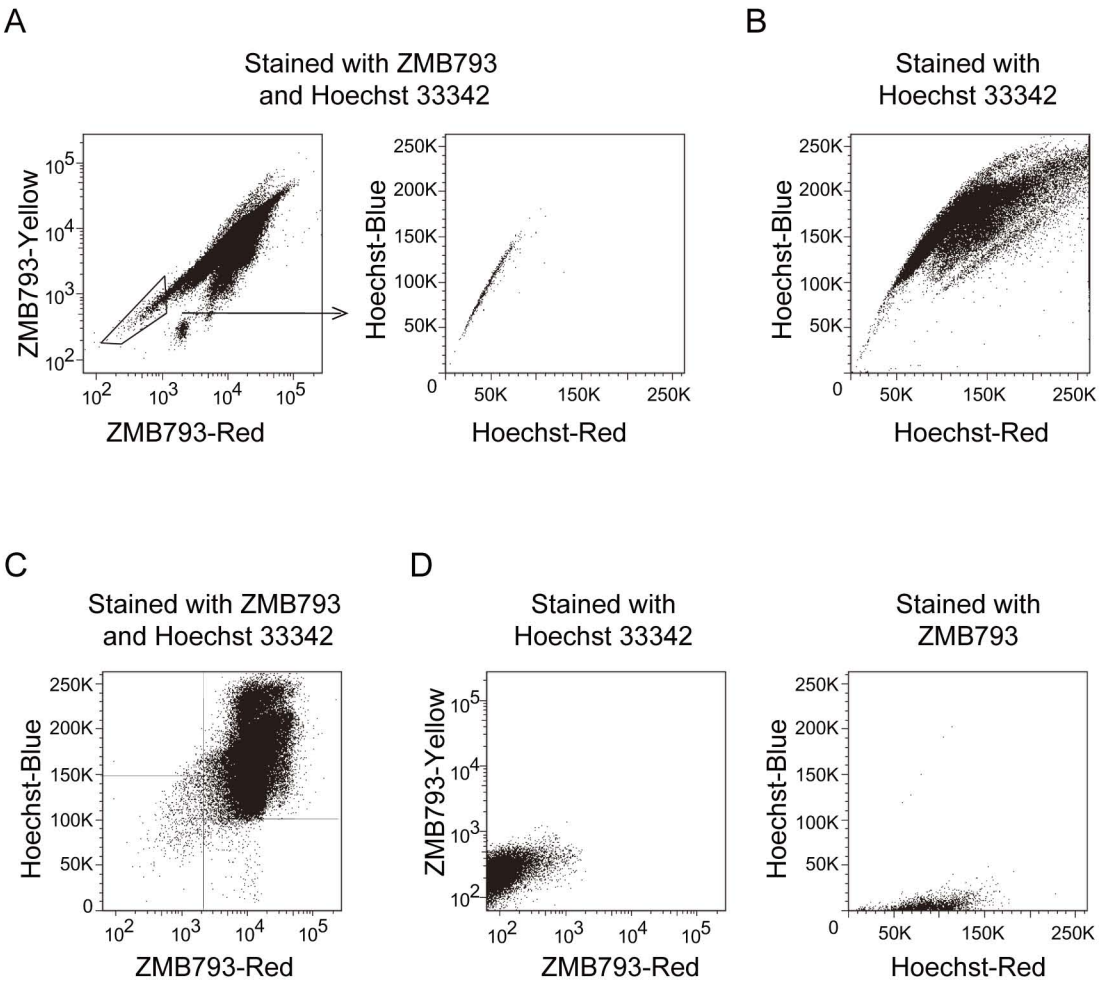

Fig. 4

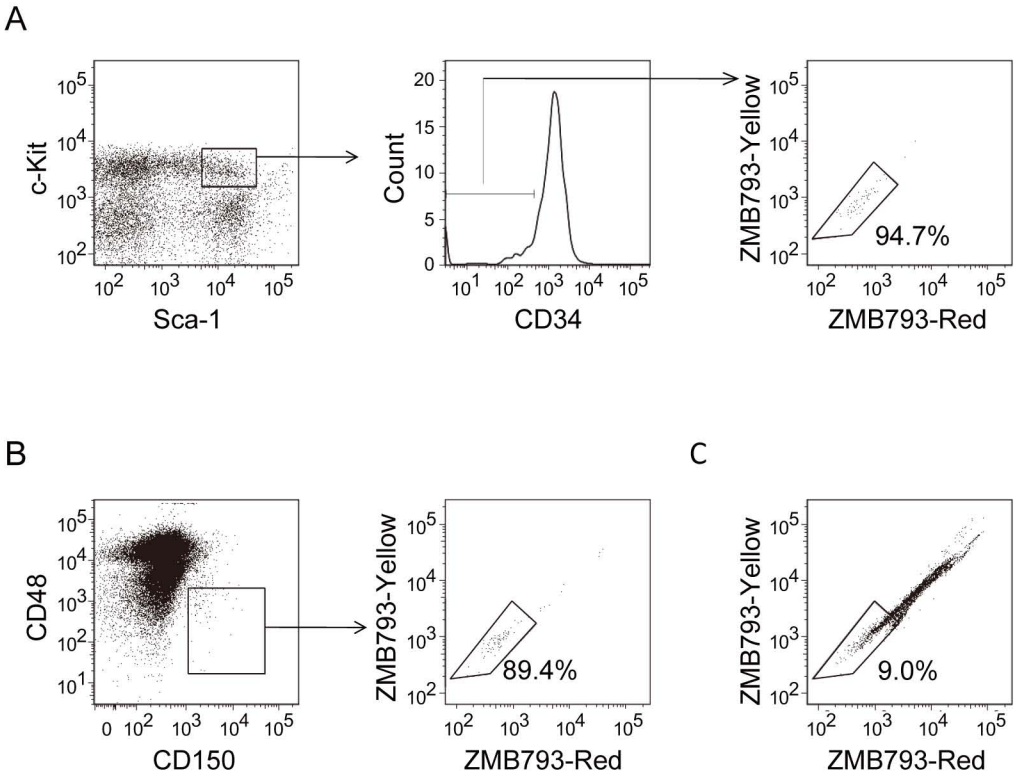

Fig. 5

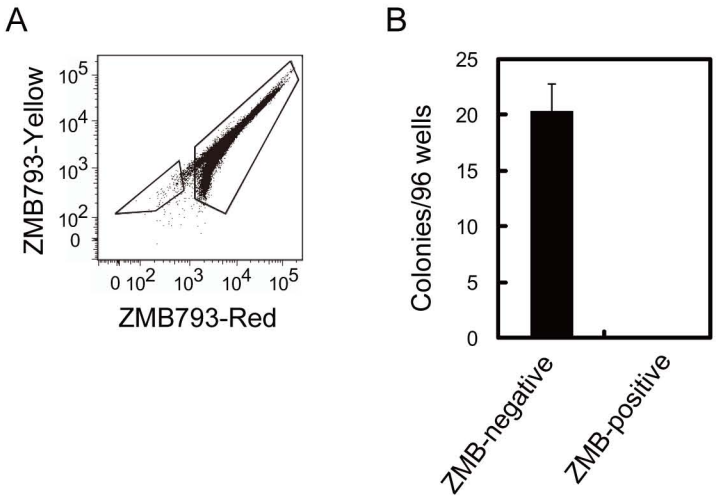

Fig. 6

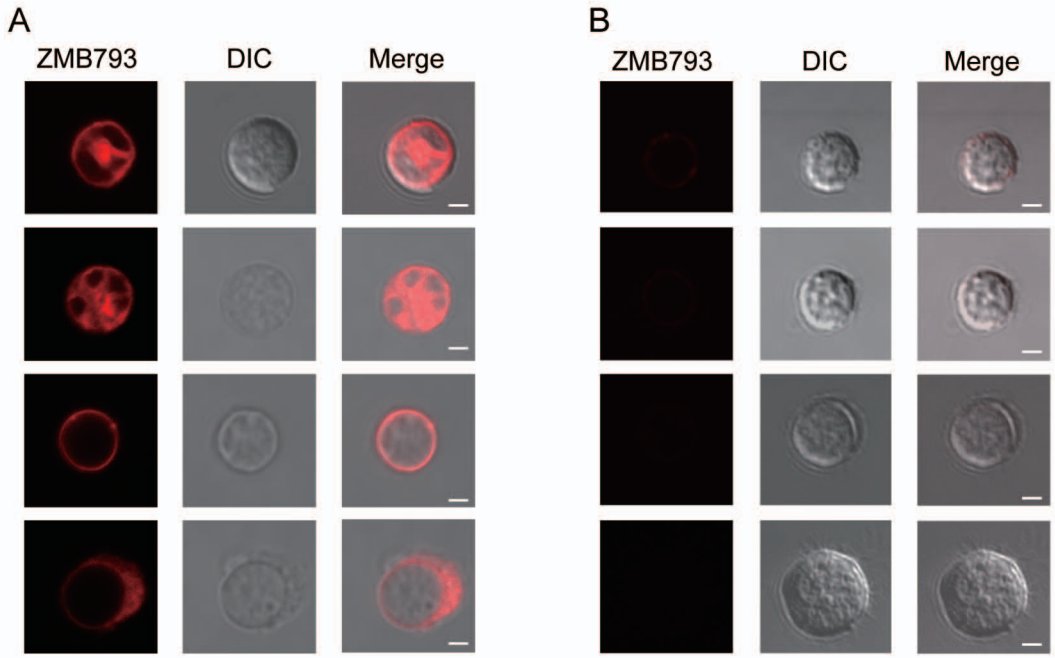

Fig. 7

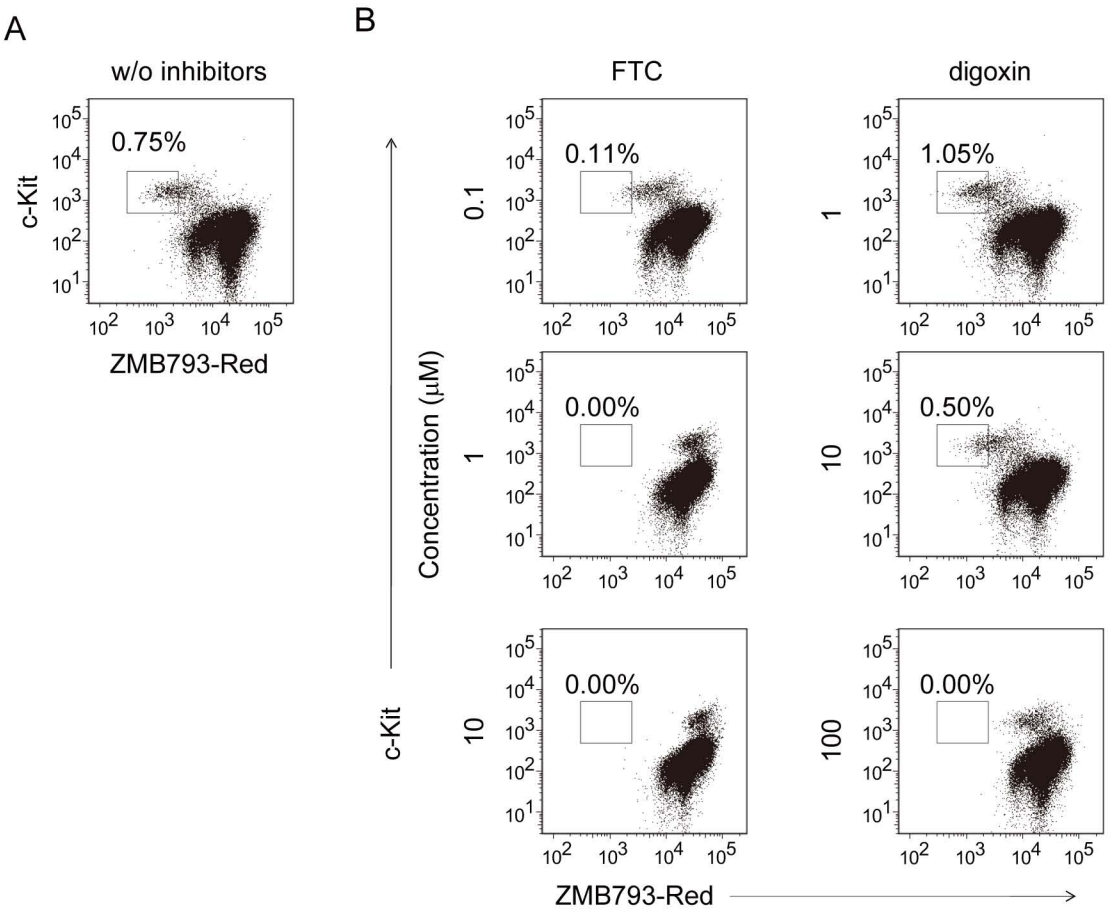

Fig. 8